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Author: Laura Lombana Sara Ortega-Atienza Julián Gómez-Gutiérrez Belén Yélamos Darrell L. Peterson Francisco Gavilanes

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The deletion of residues 268-292 of E1 impairs the ability of HCV envelope proteins to induce pore formation

Laura Lombana[a], Sara Ortega-Atienza[a1], Julián Gómez-Gutiérrez[a], Belén Yélamos[a], Darrell L. Peterson[b], Francisco Gavilanes[a*] pacog@bbm1.ucm.es

[a]Department of Biochemistry and Molecular Biology, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, 28040 Madrid

[b]Department of Biochemistry and Molecular Biology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, 23298

*Corresponding author at: Departamento de Bioquímica, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, 28040, Madrid, Spain. Tel: (+34) 91 394 42 66; Fax: (+34)91 3944159

[1]Present address: Department of Pathology and Laboratory Medicine, Brown University, Providence, RI, 02912 USA

Highlights

- HCV E2E1$_{\Delta 268\text{-}292}$ chimera, lacking the E1 putative fusion peptide, has been obtained
- The mutant chimera has the conformational features of the full length one
- HCVpp containing deleted E1 are not able to infect Huh7 cells
- The involvement of the region 268-292 in membrane pore formation is suggested

Abstract

We have obtained a chimeric protein containing the ectodomains of hepatitis C virus (HCV) envelope proteins but lacking the region 268-292 of E1. All its structural properties are coincident with those of the corresponding full length chimera. The deleted and entire chimeras were compared in terms of their membrane destabilizing properties. No differences were found in their ability to induce vesicle aggregation and lipid mixing but the deleted chimera showed a reduced capacity to promote leakage. The role of the deletion was also studied by obtaining HCV pseudoparticles (HCVpp). Both E1 and E2, and also the E1 deleted mutant, were incorporated into HCVpp to a similar level. However, HCVpp containing the E1 deleted protein are almost unable to infect Huh7 cells. These results point to the involvement of the region 268-292 in the formation of pores in the membrane necessary for the complete fusion of the membranes.

Abbreviations

HCV**:** hepatitis C virus

HCVpp**:** hepatitis C virus pseudoparticles

E2E1**:** chimeric protein containing the ectodomains of E2 and E1 HCV envelope proteins

$E2E1_{\Delta 268\text{-}292}$**:** chimeric protein containing the ectodomains of E2 and E1 but lacking the region 268-292 of E1

CCA**:** Convex Constraint Analysis

PG**:** egg yolk phosphatidylglycerol

NBD-PE**:** N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidyl-ethanolamine

Rh-PE**:** N-(lissamine rhodamine B sulphonyl) diacylphosphatidyl-ethanolamine

ANTS**:** 8-aminonaphthalene-1,3,6-trisulphonic acid

DPX**:** *p*-xylenebis-(pyridinium) bromide. LEL-CD81**:** large extracellular loop of CD 81

Keywords: hepatitis C virus; viral envelope proteins; lipid-protein interaction; membrane fusion; protein spectroscopic properties.

1. Introduction

Hepatitis C Virus (HCV) is the major cause of acute hepatitis and chronic liver disease, being the leading cause of liver transplantation in the developed world (Alter, 2006). Currently there is no vaccine available against HCV and the present standard therapy consists of α–interferon and ribavirin. However, only 10-20% of patients respond to interferon treatment and 54-56% to the combined therapy (Ronn and Sandstrom, 2008). Recently, new drugs are being used to treat HCV infection which has led to new treatments with a high efficacy but also with a high cost which precludes their use in a global scale (Kohli et al., 2014). Thus, it is clear the importance of obtaining a vaccine for HCV. In this search, a possible strategy would be to abolish the initial steps of the HCV infection. In this regard, HCV entry is a highly orchestrated process mediated by the viral envelope glycoproteins and several host factors (reviewed in (Ploss and Dubuisson, 2012)).

HCV is an enveloped, positive-stranded RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family (Lemon et al., 2007; Lindenbach and Rice, 2001). The HCV envelope glycoproteins, E1 and E2 are released from the polyprotein coded by the HCV genome after cleavage by host-cell endoplasmic reticulum proteases in positions 191/192, 383/384 and 746/747 of the polyprotein sequence, respectively (E1 corresponds to residues 192-383 and E2 to 384-746) (Mizushima et al., 1994). These glycoproteins play an essential role in virus entry into host cells by interacting with cell surface receptors and inducing fusion between the viral and cellular membranes (Bartosch et al., 2003; Nielsen et al., 2004).

Studies concerning the entry mechanism carried out with HCV isolated envelope proteins are scarce. Both E1 and E2 have been proposed as candidates for the fusion process (Drummer et al., 2007; Flint et al., 2004; Yagnik et al., 2000). In

relation to the location of the regions involved in this process, some E1 and E2 peptides with a potential fusogenic activity have been identified and characterized (Pacheco et al., 2006; Perez-Berna et al., 2006; Perez-Berna et al., 2009). A directed mutagenesis study with the HVCpp system has identified three important regions for the fusion (Lavillette et al., 2007). These are located in E1 (residues 272 to 287) and E2 (residues 419 to 433 and 597 to 620). These data suggest that different E1 and E2 regions can cooperate for the fusion process. In this sense, we have recently shown that a recombinant chimeric protein composed of E1 and E2 ectodomains possesses the membrane destabilization properties ascribed to a fusion protein (Tello et al., 2014).

In order to shed light into HCV cell entry mechanism as well as into the protein and amino acid sequences involved on it, we have studied the fusion properties of a chimeric protein, $E2_{661}E1_{340\Delta 268\text{-}292}$ ($E2E1_{\Delta 268\text{-}292}$), containing the E2 and the E1 ectodomains connected by a small hydrophilic peptide but missing a region of E1 which is supposed to contain a fusion peptide. The protein is able to accomplish the steps which are essential for fusion (Bentz et al., 1985). Also, HCVpp containing the deleted protein have been obtained and their infection capacity studied. The differences observed with respect to the intact recombinant protein point to the involvement of the region 268-292 in the fusion between the viral and cellular membranes.

2. Materials and methods

2.1. *Plasmid constructs*

The mutant cDNA $E2_{661}E1_{340\Delta 268\text{-}292}$ (hereafter $E2E1_{\Delta 268\text{-}292}$) was obtained by the method described by Pogulis (Pogulis et al, 1996). The regions on both sides of the deletion were amplified separately by PCR, using the plasmid pAcGP67A-

$E2_{661}E1_{340}$ (Tello et al., 2014) as template. The sequence of E1 and E2 corresponds to an HCV1 strain, genotype 1a (Accession number M62321). Primers GP67F (CTT GTC GGG TTT TCT CCC AGG CGC C) and GP67R (GGG TTT AAC ATT ACG GAT TTC), flanking the $E2_{661}E1_{340}$ cDNA in the pAcGP67-A plasmid, were used as external primers. Internal primers of the mutant had a complementary region of 18 nucleotides, corresponding to the 9 final nucleotides and the first 9 nucleotides located before and after the deletion:

Primer E1Δ268F: CTT GTC GGG TTT TCT CCC AGG CGC C

L_{265} V_{266} G_{267} F_{293} S_{294} P_{295} R_{296} R_{297}

Primer E1Δ268R: GGG AGA AAA CCC GAC AAG CAG ATC G

P_{295} S_{294} F_{293} G_{267} V_{266} L_{265} L_{264} D_{263}

After co-purification of both PCR products, $E2E1_{192\text{-}267}$ and $E1_{293\text{-}340}$, they were subjected to a second elongation PCR without primers in order to expand the area. Finally, a third PCR using the elongated sequence as template, and the oligonucleotides GP67F and GP67R as primers, was performed to amplify the mutant $E2E1_{\Delta 268\text{-}292}$ cDNA fragment. This was subsequently digested with restriction enzymes *Bam*HI and *Not*I and cloned in the pAcGP67-A baculovirus transfer vector (Pharmigen) to generate the recombinant plasmid pAcGP67-A-$E2E1_{\Delta 268\text{-}292}$.

2.2. Protein expression and purification

$E2E1_{\Delta 268\text{-}292}$ was expressed in baculovirus-infected High Five insect cells and the secreted protein was purified from the extracellular medium by Ni^{2+}-nitrilotriacetic acid-agarose chromatography following the methodology previously described for the intact chimera E2E1 (Tello et al., 2015).

2.3. Protein analysis

Protein concentration was determined spectrophotometrically from the absorbance at 280 nm and the extinction coefficient calculated from amino acid analysis. The absorption spectra were recorded on a Beckman DU-640 spectrophotometer. The amino acid analysis of hydrolyzed aliquots was performed on a Beckman 6300 automatic amino acid analyzer.

2.4. Circular Dichroism

CD measurements were carried out at room temperature on a Jasco spectropolarimeter, model J-715. The secondary structure of the protein was evaluated by computer fit of the dichroism spectra according to Convex Constraint Analysis (CCA) (Perczel et al., 1991).

2.5. Fluorescence spectroscopy

Emission spectra were obtained using an SLM AMINCO 8000C spectrofluorimeter. Excitation was performed at 275 or 295 nm, and the emission spectra were recorded over the range 285-450 nm. The tyrosine contribution to the emission spectra was calculated as previously described (Tello et al., 2015).

2.6. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli using 15% polyacrylamide gels (Laemmli, 1970). The molecular mass of the protein bands was estimated by comparison with

protein markers of known molecular mass (Prestained SDS-PAGE Standards, Bio-Rad).

2.7. Western blotting

After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham) in 48 mM Tris-HCl pH 9.0, containing 39 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol, for 1 h at 1 mA/cm^2, by using a V20-SDB apparatus (Scie-Plas). To detect proteins, membranes were incubated for 16 h at 4 ºC either with a HRP-conjugated monoclonal anti-His antibody (Sigma) at a 1:3000 dilution, with the A4 monoclonal anti-E1 antibody at a 1:1000 dilution or with a mixture of monoclonal antibodies anti-E2 ALP98 (1 ug/ml) and AP33 (1:1000). All the anti-E1 and anti-E2 monoclonal antibodies were kindly provided by Dr. Jean Dubuisson, Institute Pasteur de Lille, France. In the case of incubations with anti-E2 and anti-E1 antibodies, membranes were washed and incubated for 1 h at room temperature with the corresponding secondary antibody, goat anti-mouse IgG (Sigma) at a 1:3000 dilution. Membranes were developed with ECL reagents (Amersham Life Sciences) and exposure using the program LaserJet3000.

2.8. Enzyme Linked Immunosorbent Assay (ELISA)

The reactivity of the recombinant proteins against human sera was tested as previously described (Tello et al., 2015). Six HCV-positive and two HCV-negative human sera, diluted between 1:100 and 1:500, were used. Bound antibodies were detected using HRP-conjugated anti-human IgG (Fc). Human sera were provided by Dr. Fernando Vivanco (Fundación Jiménez Díaz, Madrid, Spain).

For H53 ELISA assays, the same protocol was followed but with these exceptions but the plates were coated with 1 µg/well of purified protein. E2 was detected with H53 antibody at a 1:400 dilution and a goat anti-mouse-HRP conjugated antibody (Sigma) at a 1:3000 dilution. The monoclonal antibody H53 is conformation-dependent and was kindly provided by Dr. Jean Dubuisson (Institute Pasteur de Lille, France).

2.9. LEL-CD81 binding assay

Binding of E2E1 and E2E1$_{\Delta 268\text{-}292}$ to the large extracellular loop of human CD81 (LEL-CD81) was determined as previously described (Ortega-Atienza et al., 2014). Briefly, 0.3 µg of a glutathione S-transferase fusion protein containing the second extracellular region (EC2) of the LEL of human CD81 (Higginbottom et al., 2000) were used to coat Nunc F96 Maxisorp, 96 well-plates. Then the entire or the deleted soluble chimera at different concentrations were added and incubated at room temperature for 2 h. CD81-bound protein was detected using a polyclonal antibody raised against isolated E2 ectodomain obtained in our laboratory followed by a goat anti-rabbit IgG (whole molecule)–peroxidase antibody (1:3000) (Sigma).

2.10. Vesicle preparation

The vesicles used were prepared by sonication and extrusion in a Liposo Fast-Basic extruder apparatus (Avestin, Inc.) with 100-nm polycarbonate filters (Costar) as previously described (Tello et al., 2014). Lipid concentration was calculated based on their phosphorous content determined according to Barlett (Barlett, 1959).

2.11. Aggregation studies

The aggregation of phospholipid vesicles induced by the addition of the recombinant proteins was followed by measuring the optical density at 360 nm (ΔOD_{360}) on a Beckman DU-640 spectrophotometer after incubation for 1 h at 37 ºC (Tello et al., 2014). The final phospholipid concentration was 0.14 mM. In all cases, controls of lipid vesicles in the absence of protein and protein in absence of lipid were obtained.

2.12. Lipid mixing assay

The fluorescent probe dilution assay (Struck et al., 1981), was employed to determine lipid mixing. All the conditions are coincident with those previously used for $E1_{340}E2_{661}$ (Tello et al 2014). The concentrations of the donor (NBD-PE) and acceptor (Rh-PE) were 0,11 and 0,06 μM, respectively. The efficiency of fluorescence resonance energy transfer (%RET) was calculated from the ratio of the intensities at 530 and 590 nm and the appropriate calibration curve which was obtained by using labeled vesicles containing 0,11 μM NBD-PE and variable concentrations of Rh-PE (between 0 and 0,075 μM). The final phospholipid concentration was 0.14 mM.

2.13. Release of aqueous contents

Leakage was determined by the ANTS/DPX leakage assay (Ellens et al., 1985) as previously described (Tello et al., 2014). ANTS and DPX were co-encapsulated in phospholipid vesicles at 12.5 mM and 45 mM respectively, in 10 mM Tris pH 7.2, 20 mM NaCl. The fluorescence spectra were measured in a SLM Aminco 8000C spectrofluorimeter. The excitation wavelength was set at 385 nm and the ANTS emission was monitored at 520 nm. The fluorescence of control vesicles

without protein was taken as 0% leakage while 100% was considered upon addition of 0.5% Triton X-100.

2.14. Production and Detection of HCV pseudoparticles

The HCV pseudoparticles (HCVpp) were obtained by a previously reported method (Bartosch et al., 2003). The plasmids pMLV-Gag-Pol, which allows the expression of retroviral structural proteins, and pMLV-Luc, which codes the luciferase, were kindly donated by Dr. Cosset of the École Normal Supérieure de Lyon. pcDNA-$C_{132}E1_{\Delta 268-292}E2$ was constructed following the procedure described above for plasmid pAcGP67-A-$E2E1_{\Delta 268-292}$ using the same primers and the plasmid cDNA-C_{132}E1E2 as template. The later was previously obtained in our laboratory by cloning directly the sequence of the HCV DNA into pcDNA3.1 plasmid between *Eco*RI and *Xba*I sites and contains the sequence which codes from residue 132 of the core protein and the entire E1 and E2 sequences including the transmembrane domains of both as they are in the original cDNA. In this case, the primers used for the ends are pENF-1 (F): 5'- GGT GGG AGG TCT ATA TAA -3' and pcDNA-BGH (R): 5'- GGA GGG GCA AAC AAC AGA TG -3', which hybridizes 159 bp before *Eco*RI an 30 bp after *Xba*I, respectively.

To generate HCVpp, HEK 293T cells were transfected with expression vectors encoding the viral components. Briefly, the Gag-Pol packaging construct (8 µg), the luciferase vector construct (8 µg), and the glycoprotein-expressing construct (3 µg) DNAs were transfected into $8x10^5$ HEK 293T cells seeded the day before in 10 cm plates using a calcium phosphate transfection protocol (CLONTECH Laboratories, Inc.), as described (Bartosch et al., 2003). The medium (10 ml/plate) was replaced 16 h after transfection by 5.5 ml/plate with 10mM HEPES pH 7.0. Supernatants containing the pseudoparticles were harvested 24 h later, filtered through 0.45 µm

pore-sized membranes, and used in entry assays. Purified pseudoparticles were obtained by ultracentrifugation of 10 ml supernatants through a 1.5 ml 20% sucrose cushion in an SW 41 Beckman rotor (30000 rpm, 127 min, 4 ºC). The pellets were suspended in 20 µl loading buffer. Immunoblots of HCV expressing cell lysates and purified pseudoparticles were performed as described above.

2.15. Infection Assays

Huh7 target cells were seeded in 24 well plates at a density of $3x10^4$ cells per well and incubated overnight at 37 ºC. 150 µl of supernatants containing the pseudoparticles were added to the cells and the plates were incubated for 4 h. The supernatants were removed and the cells were incubated in regular medium for 84 h at 37 ºC. The luciferase activity was then measured using the Bright-Glo luciferase kit (Promega) in a LB9507 luminometer (Thermo Fisher). The infectious titers are expressed as relative luminescence units.

3. RESULTS

3.1. Expression and purification of $E2E1_{\Delta 268-292}$

Fig. 1 shows a scheme of $E2E1_{\Delta 268-292}$. The recombinant chimera would have 432 amino acids, 278 corresponding to positions 384–661 of E2 ectodomain, 124 corresponding to positions 192–340 of E1 ectodomain without the fusogenic peptide, and the rest being due to the cloning strategy, that is the spacer, the TEV sequence between E1 and E2 and the His tag. Following the methodology recently described for the entire $E2_{661}E1_{340}$ chimera (Tello et al., 2015), the mutant protein was expressed in High Five™ insect cells transfected with the pAcGP67-A-$E2E1_{\Delta 268-292}$ plasmid. The protein was secreted to the extracellular media and subsequently purified by affinity chromatography using a Ni-nitrilotriacetic acid-agarose column.

The entire $E2_{661}E1_{340}$ was also purified and used as a control in all the experiments. Following this procedure, approximately 11 mg of highly pure $E2E1_{\Delta 268-292}$ protein were obtained from 1 L of culture media.

3.2. Biochemical characterization of $E2E1_{\Delta 268-292}$

The amino acid composition of the recombinant protein determined by amino acid analysis was coincident with that deduced from the cDNA sequence (data not shown). The extinction coefficient at 280 nm calculated from the absorption spectrum and using the protein concentration calculated from the amino acid analysis was 21795 $M^{-1}cm^{-1}$. This value together with the absorbance at 280 nm allowed the determination of the protein concentration in all subsequent experiments.

The recombinant protein behaves as a single band in SDS-PAGE in the presence of reducing agents. The calculated molecular mass, 64.4 kDa, was higher than that expected from the amino acid sequence (47.9 kDa) which indicates that the protein was highly glycosylated in insect cells (Fig. 2, lanes 3 and 4). In the absence of reducing agents, dimers, trimers and higher order oligomers are observed for both entire and deleted chimeras (Fig. 2a, lanes 1 and 2). Moreover, both proteins were recognized by a monoclonal anti-E1 antibody, a mixture of monoclonal AP33/ALP98 anti-$E2_{661}$ antibodies as well as a monoclonal anti-His antibody (Fig. 2b).

The spectroscopic characterization of $E2E1_{\Delta 268-292}$ was carried out by means of circular dichroism and fluorescence spectroscopies. The spectra were taken in parallel with E2E1 in order to check if deletion of 25 amino acids of E1 induced any conformational change.

The far-UV CD spectrum of both proteins showed a minimum at 208 nm, with a residue molar ellipticity of -7200 degree·cm^2·$dmol^{-1}$, indicative of a high level of aperiodic structure (Fig. 3a). Deconvolution of these spectra using the program CCA (Perczel et al., 1991) yielded the following percentages of secondary structure elements: α helix 13 and 14%, β sheet 48 and 45% and non-ordered structures 39 and 41% for E2E1 and $E2E1_{\Delta 268\text{-}292}$, respectively. Thus, both proteins showed a very similar secondary structure pattern, with a low helical content being the β-sheet the main ordered structure. These results show that there are no changes in terms of secondary structure when the amino acids 268-292 are deleted from the chimera E2E1.

Fluorescence emission spectrum of $E2E1_{\Delta 268\text{-}292}$, as well as of E2E1, showed a maximum at 333 nm upon excitation at 275 nm and 295nm (Fig. 3b). In both cases, the contribution of tyrosine residues to the emission fluorescence upon excitation at 275 nm was very low, approximately 5 % (Fig. 3b). Thus, we can conclude that in both proteins the Trp residues are located in a very similar environment of relatively low hydrophobicity. The spectroscopic data indicate that the global structure of both proteins is similar and that the deletion of the putative fusion peptide is not relevant for the folding of the protein.

3.3. Antigenic characterization of $E2E1_{\Delta 268\text{-}292}$

A panel of six HCV-positive and two HCV-negative human sera was used to assess the antigenic properties of $E2E1_{\Delta 268\text{-}292}$ in comparison with E2E1. Both proteins were able to bind to antibodies present in the HCV-positive human sera (Fig. 4, sera 1-6) at different levels, whereas they were not recognized by immunoglobulins from HCV-negative sera (Fig. 4, sera 7 and 8). The reactivity pattern was very similar in both cases. However, it must be noted that the mean

signal obtained with E2E1$_{\Delta 268\text{-}292}$ is about 37% higher than the observed with E2E1. This result could indicate that the antigenic determinants are more exposed in the recombinant protein with the putative fusogenic peptide deleted. Anyhow, it is clear that the overall reactivity is maintained.

On the other hand, the reactivity of both proteins with the anti-E2 conformation-dependent H53 monoclonal antibody was very similar (Fig. 4, inset). Again, these results suggest that both, E2E1 and E2E1$_{\Delta 268\text{-}292}$, possess very similar conformations with some differences in the degree of exposure of some epitopes. Moreover, both E2E1 and E2E1$_{\Delta 268\text{-}292}$ were able to bind to CD81 in a concentration dependent manner, being the reactivity of both proteins very similar.

3.4. Membrane destabilization properties of E2E1$_{\Delta 268\text{-}292}$

The ability of the recombinant E2E1$_{\Delta 268\text{-}292}$ protein to induce vesicle aggregation of PG liposomes was monitored by measuring the increase in the optical density at 360 nm (ΔOD_{360}) as a result of the enlargement in vesicle size upon incubation with different concentrations of E2E1$_{\Delta 268\text{-}292}$ and E2E1. The results obtained for the different phospholipid/protein ratios at pH 5.0 and pH 7.0 are depicted in Fig. 5(a). E2E1$_{\Delta 268\text{-}292}$ was able to generate slightly higher levels of aggregation than E2E1 showing the same pH and concentration dependence. The observed pH dependence would be a consequence of the viral fusion taking place at acidic pH in the endosomes (Lavillette et al., 2006). Thus, the region between 268 and 292 in E1 seems not to be relevant for the aggregation process.

As it is observed in Fig. 5(b), both E2E1$_{\Delta 268\text{-}292}$ and E2E1 proteins were also able to induce lipid mixing of PG vesicles, both at pH 7.0 and 5.0, being the effect produced at neutral pH much lower than that observed at acidic pH. At pH 5.0,

%RET decreased from 63%, in the absence of protein, to around 18% at a protein/lipid ratio of 20 x 10^{-3}. This value corresponds to a 3.5 fold dilution in acceptor surface density. This fact together with the considerable increase in vesicle size stated above would support the almost complete fusion of the vesicles (Ellens et al., 1985). When neutral pH was used, the energy transfer only diminished from 60% to 42-47% which again points out for a fusion process taking place at the endosomes. Since the levels reached by both proteins are very similar, the putative fusion peptide would not have either a role in this step of the fusion process.

Fig. 5(c) shows the leakage induced by the recombinant proteins when they were added to PG vesicles at pH 5.0. Both E2E1 and E2E1$_{\Delta 268\text{-}292}$ were able to promote the release of internal contents of the vesicles in a dose-dependent manner. However, the putative fusion peptide seems to have an effect in this fusion step since a difference in the protein concentration needed to attain the same level of leakage was observed. Thus, when E2E1 was employed, the 80% of leakage was reached at a protein/phospholipid ratio of 0.25 x 10^{-3} but when E2E1$_{\Delta 268\text{-}292}$ was used, a protein concentration 15 times higher was needed to attain similar leakage levels.

3.5. Entry capacity of HCVpp containing the mutant protein

The role of deleting the E1 putative fusion peptide on the function of the envelope glycoproteins has been studied by means of HCVpp. They were obtained by transforming HEK 293T cells with the plasmid pcDNA-C_{132}E1$_{\Delta 268\text{-}292}$E2. Also, HCVpp without envelope proteins and those containing C_{132}E1E2 were obtained and used as negative and positive controls, respectively. The expression and integration of the envelope proteins were evaluated by immunodetection both in lysed HEK 293T transformed cells and in purified particles analyzed by SDS-PAGE, respectively (Fig. 6). The expression of both glycoproteins is obvious in transfected cells with both

plasmids but no bands are observed when the cells were transformed with the plasmid lacking the envelope proteins (Fig. 6b). To detect the presence of E1 and E2 in isolated pseudoparticles, they had to be concentrated by ultracentrifugation. It can be seen that both glycoproteins are incorporated into HCVpp of both types and that the levels of expression are very similar (Fig. 6a).

The HCVpp were used to infect Huh7 cells. The luminescence produced by the luciferase after four days of incubation was used to quantify the infectivity level because only the pseudoparticles able to enter the cells would lead to a luminescence signal. When the cells were infected with HCVpp $C_{132}E1_{\Delta 268\text{-}292}E2$ the signal decreased to 4.8±1.1 as compared to the positive control (100.0±1.0). The luminescence signal obtained with HCVpp containing no envelope proteins was 1.8±1.0. Since the expression and incorporation levels are similar the decrease of the infectivity could be attributed to the absence of the putative fusion peptide in particles produced in pcDNA-$C_{132}E1_{\Delta 268\text{-}292}E2$ transfected cells.

4. DISCUSSION

The difficulty of expanding HCV in cell culture has hampered for many years functional studies on HCV infection. The cellular mechanism of HCV entry has been studied using HCV pseudoparticles (HCVpp), infectious retroviral particles with HCV envelope proteins on the surface, and the cell culture model which allows the production and propagation of virus in cell culture (HCVcc) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Reports using both HCVpp and HCVcc models have evidenced the pH sensitivity of HCV entry (Blanchard et al., 2006; Haid et al., 2009; Lavillette et al., 2006). Several studies suggest that HCV enters cells by clathrin-mediated endocytosis (Blanchard et al., 2006) and that fusion occurs in the early endosomes (Meertens et al., 2006). Furthermore, the acidic pH of endosomes

triggers the fusion process probably by inducing conformational changes in the envelope proteins (Blanchard et al., 2006; Koutsoudakis et al., 2006; Meertens et al., 2006; Tscherne et al., 2006).

The exposed nature of the hepatitis C virus envelope proteins makes them good candidates to participate in the fusion between the viral and cellular membranes. In fact, bioinformatics studies suggest that both E1 and E2 are class II fusion proteins (Garry and Dash, 2003; Yagnik et al., 2000) although recent structural studies of E1 and E2 fragments do not support this idea (El Omari et al., 2014; Khan et al., 2014; Kong et al., 2013). However, several studies suggest the involvement of both E1 and E2 in the process (Drummer et al., 2007; Flint et al., 2004; Yagnik et al., 2000). On the other hand, the data concerning the location of the regions with fusogenic properties are limited and entail amino acid sequences from both E1 and E2 glycoproteins. Thus, several membrane active regions have been found in E1 and E2 which suggest that both proteins participate in the fusion process (Pacheco et al., 2006; Perez-Berna et al., 2006). A peptide derived from the 265-296 amino acid sequence of E1 has been synthesized and its membrane destabilizing properties determined (Perez-Berna et al., 2009). Other group has identified six regions in E1 and E2 with features of fusion peptides and has proposed that at least the regions 270-284 of E1 and 416-450 and 600-620 of E2 participate in the membrane fusion process (Lavillette et al., 2007). Mutation analysis of hydrophobic residues in the putative fusogenic region of E1 has revealed that only the F285A mutant abolished viral entry whereas other mutations did not affect or only partially inhibited viral entry (Drummer et al., 2007). Finally, it has been reported that specific residues between positions 264 and 290 within E1, but not the structure, are responsible for fusion (Li et al., 2009).

Few studies on the involvement of isolated envelope proteins in HCV entry mechanism have been published. In an effort to contribute solutions to the location of the fusion region, we have obtained a chimeric protein, $E2E1_{\Delta 268-292}$, where one of the putative fusion peptides has been eliminated. The amino acid sequence analysis of E1 and E2 indicates that they are type-I transmembrane proteins, highly glycosylated, with an N-terminal ectodomain and a C-terminal hydrophobic domain anchoring these glycoproteins to the membrane. Moreover, it has been shown that E1 folding is dependent on E2 co-expression (Michalak et al., 1997; Patel et al., 2001), and that E2 folding is also dependent on E1 co-expression (Brazzoli et al., 2005; Cocquerel et al., 2003).

We have considered the presence of both ectodomains in a single polypeptide chain because heterodimerization of the E1/E2 glicoproteins has been described to be mandatory for fusion of viral and host cell membranes during the infection of HCV (Ciczora et al., 2007; Op De Beeck et al., 2001; Op De Beeck et al., 2004). Also, the presence of both proteins is necessary to form infectious pseudoparticles (Sandrin et al., 2005). We have previously obtained a chimera, $E1_{340}E2_{661}$, composed of the ectodomains of E1 and E2 (Tello et al., 2010) and shown that it possesses fusogenic properties (Tello et al., 2014). Moreover, we have also shown that the two domains can be permuted so that the recombinant protein $E2_{661}E1_{340}$ is structurally indistinguishable of its counterpart $E1_{340}E2_{661}$ but the exchange process leads to a 4 times increase of the expression yield (Tello et al., 2015). Also, the properties of E2E1 shown throughout this paper confirm the identity of both original and permuted chimeras in terms of membrane destabilization properties. Thus, we have used the later to obtain a mutant where the E1 fusogenic region has been deleted. Based on the interfacial hydrophobicity (Wimley and White,

1996) and hydropathicity profiles (Kyte and Doolittle, 1982) we decided to delete the region 268-292 that, although does not exactly coincide with published regions, it contains the putative E1 fusogenic stretch described by others (Lavillette et al., 2007; Perez-Berna et al., 2009).

The properties of the mutated chimera $E2E1_{\Delta 268-292}$ are practically coincident with those of the entire protein. Thus, all the spectroscopic features such as dichroism and fluorescence spectra revealed that the protein is correctly folded and has the structure of a native protein with the same properties than the complete chimera E2E1. With respect to the oligomerization properties, the mutated and entire proteins behave as dimers, trimers and higher order oligomers in the absence of reducing agents. Although it has been described that the large covalent E1E2 complexes which can be observed in PAGE-SDS gels correspond to nonfunctional aggregates (Op De Beeck et al., 2004), more recent studies, using the HCVcc system demonstrated that the large E1E2 disulfide-linked complexes maintain a native conformation (Vieyres et al., 2010). Then, the disulfide-bridge oligomerization of E2E1 and $E2E1_{\Delta 268-292}$ seems to be compatible with the maintenance of its native properties.

Antigenic studies revealed that both, the complete chimera and the mutated one are equally recognized by antibodies present in HCV positive sera. For most of the sera, the deletion is accompanied by a slightly higher degree of exposure of the antigenic determinants which gives rise to higher absorbance values in the assay performed. Anyway, all these characteristics point to a slight modification of the overall structure as a consequence of the deletion and allow us to compare both proteins in terms of membrane destabilizing properties and extract conclusions about the role of the region 268-292 of E1 in the initial steps of the infective cycle.

As expected from previous reports (Haid et al., 2009; Lavillette et al., 2006; Tello et al., 2014), the aggregation and lipid mixing results indicate that the fusion process is pH dependent and the attained levels can be decreased by increasing the ionic strength of the medium (data not shown). On the other hand, no differences were noted when comparing the ability of E2E1 and $E2E1_{\Delta 268\text{-}292}$ to induce aggregation and lipid mixing. However, when leakage assays were compared, a drastic reduction of the capacity to disrupt the membrane was observed. A 10 times higher protein concentration is needed to attain results comparable to those of the entire chimera. As pointed out above, this reduction of the disruption ability can be attributed to the deletion of the fusion peptide itself and not to a change in the structure as a consequence of the elimination.

The role of the deletion on the function of the envelope proteins was studied by obtaining HCVpp which could give us some clue about the involvement of the removed peptide in the entry mechanism. Both E1 and E2, and also the E1 deleted mutant, were incorporated into the pseudoparticles to a similar level. However, HCVpp $C_{132}E1_{\Delta 268\text{-}292}E2$ are almost unable to infect Huh7 cells. Since the isolated E2E1 and $E2E1_{\Delta 268\text{-}292}$ proteins bind to the CD81 receptor, this diminution could be due to the reduced capacity of the mutant protein to disrupt the membrane as revealed by the leakage assay. In fact, it has been described that the fusion process comprise several steps: membrane apposition which destabilize the interface, formation of the hemifusion intermediate where mixing of the lipids can occur and finally the formation of pores and full collapse of the membranes (Sapir et al., 2008). If the mutant chimera is not able to produce pores in the membrane, this protein would not be able to fuse membranes and hence the pseudoparticles obtained with this protein would not have the capability to enter and infect cells. Anyhow, since

aggregation and lipid mixing are not changed when E1 peptide fusion is missing, some other stretches in E1 and/or E2 must be involved. In this sense, fusion must be a really complex process with the participation of several regions such it has been described in other viruses (Peisajovich and Shai, 2003) and even in HCV (Lavillette et al., 2007).

Therefore, the data presented here point to the involvement of the region 268-292 of E1 in the fusion process. In this sense it is worth mentioning that the deleted E1 fusion peptide amino acid sequence of the different HCV genotypes has common characteristics .Thus, of the 25 amino acids, 13 are fully conserved or changed by others with similar properties. Moreover, the distribution of small and hydrophobic amino acids as well as sulfhydryl, hydroxyl and glycine residues is very similar. Finally, these data also validate the use of the E2E1 chimera in the localization of the regions responsible for the fusion of the viral and cellular membranes and makes it a good candidate to be used as an immunogen in the control of the viral infection since it could produce antibodies that would block the entry of HCV into the hepatocytes.

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Figure Captions

Fig. 1. Schematic representation of $E2E1_{\Delta 268\text{-}292}$. The E1 and E2 proteins are showed in white, and the spacer-TEV region in grey. The TEV protease recognition site (ENLYPQ) is shown. The arrow indicates the cleavage site. The deleted amino acids are noted with an empty triangle. The 6xHis tag at the N terminal end is shown in black.

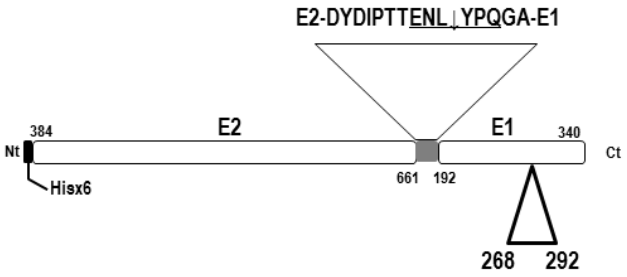

Fig. 2. Molecular characterization of $E2E1_{\Delta 268\text{-}292}$. (a) SDS–PAGE of E2E1 (lanes 1 and 3) and $E2E1_{\Delta 268\text{-}292}$ (lanes 2 and 4) purified recombinant proteins in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of 5% β-mercaptoethanol. The position of the molecular mass markers is indicated. The gel was stained with Coomassie Brilliant blue R-250. Lanes 1 to 4 belong to the same gel but some empty lanes, which were included to avoid diffusion of the reducing agent, have been removed for simplicity. (b) Western blot analysis of purified E2E1 and $E2E1_{\Delta 268\text{-}292}$ using anti E2 (AP33+ALP98), anti E1 (A4) and anti His antibodies.

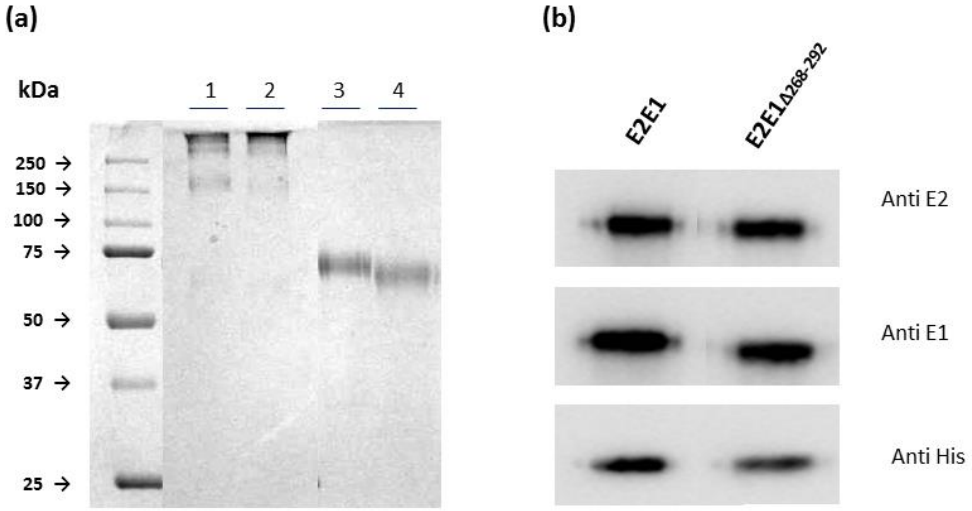

Fig. 3. Spectroscopic characterization of E2E1 and E2E1$_{\Delta 268\text{-}292}$. (a) Far-UV CD spectra at pH 8.0. The protein concentration was 0.1 mg/ml. Spectra were recorded five times, averaged and corrected for buffer contributions. Data are expressed as mean residue molar ellipticity. (b) Fluorescence emission spectra at pH 7.0 upon excitation at 275 nm. The Tyr contribution is also shown. The protein concentration was 0.05 mg/ml. The buffer employed was 20 mM Tris pH 8.0, 50 mM NaCl and its contribution was always subtracted. Spectra were collected at room temperature. The spectra shown are representative of those obtained for three different protein preparations.

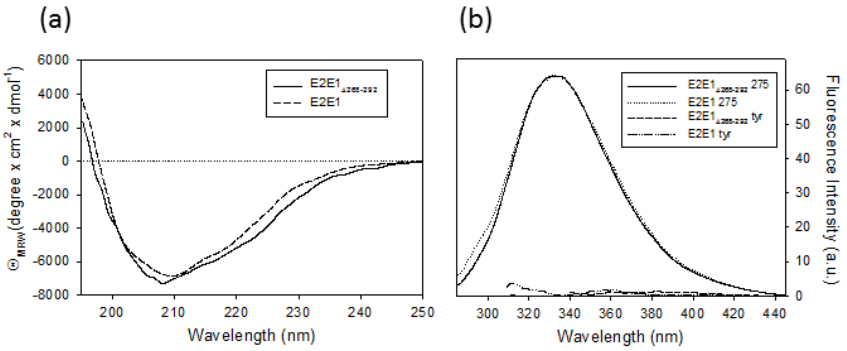

Fig. 4. Reactivity of E2E1 and E2E1$_{\Delta 268\text{-}292}$ against HCV-positive (1-6) and negative (7 and 8) human sera. 96-well plates were coated with 100 ng of purified recombinant protein/well. (Inset) Reactivity of E2E1 and E2E1$_{\Delta 268\text{-}292}$ against H53 conformation-dependent monoclonal antibody. Averages and standard deviations of three independent experiments are shown.

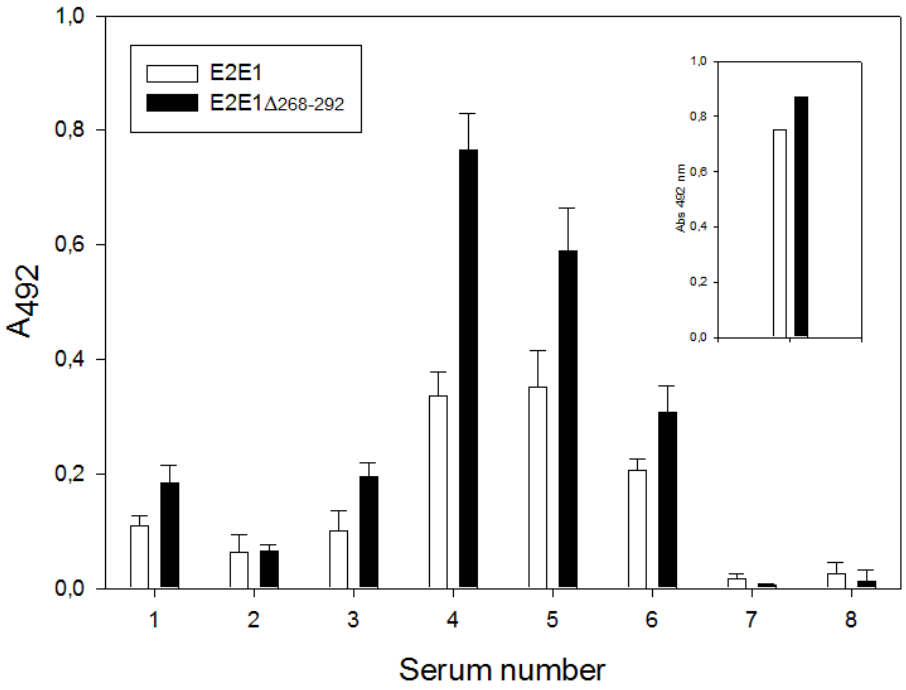

Fig. 5. Aggregation (a), lipid mixing (b) and leakage (c) of PG vesicles induced by E2E1 and E2E1$_{\Delta 268\text{-}292}$. The final phospholipid concentration was 0.14 mM. (a) The increase of the optical density at 360 nm (ΔOD_{360}) was measured after incubation of vesicles in medium buffer at pH 5.0 or pH 7.0. (b) Increasing concentrations of each protein were added to a 1:9 mixture of labeled (0,11 μM NBD-PE and 0,06 μM Rh-PE) and unlabeled PG vesicles hydrated in medium buffer at pH 7.0 or 5.0. (c) Increasing concentrations of both proteins were added to vesicles loaded with ANTS and DPX in medium buffer at 5.0. Averages and standard deviations of three independent experiments are shown.

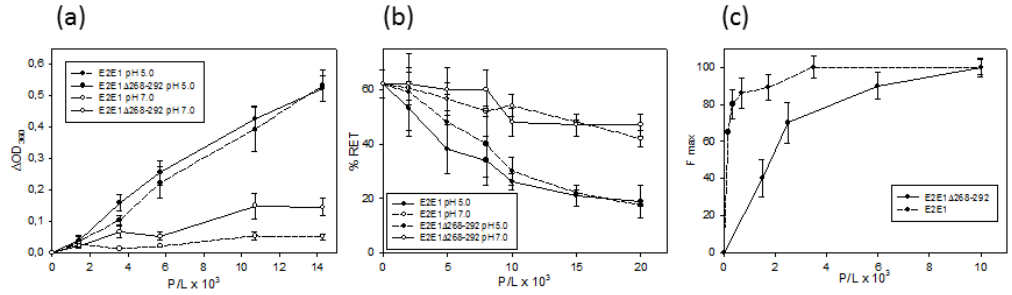

Fig. 6. Immunodetection of E1 and E2 envelope glycoproteins in purified HCVpp (a) and transfected HEK 293T cells (b). E1 was detected with A4 monoclonal antibody and E2 with a mixture of AP33 and ALP198 monoclonal antibodies. Gag protein was detected with CRL1912 rat antibody.